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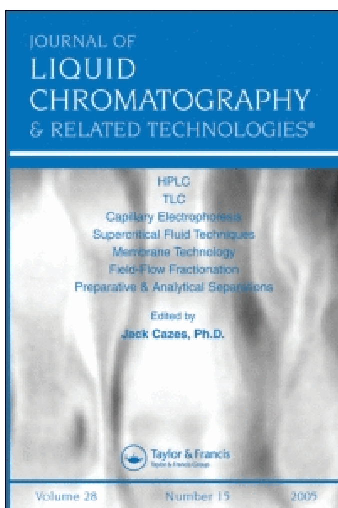

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Quantification of 4-OH-2,6-Xylidine and its Conjugates in Human Urine Samples Utilising Microextraction in Packed Syringe On-line with Liquid Chromatography and Electrospray Tandem Mass Spectrometry (MEPS-LC-MS/MS)

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Quantification of 4-OH-2,6-Xylidine and its Conjugates in Human Urine Samples Utilising Microextraction in Packed Syringe On-line with Liquid Chromatography and Electrospray Tandem Mass Spectrometry (MEPS-LC-MS/MS)

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Abstract: A method has been developed to quantify the major metabolite, 4-OH-2,6-xylidine (4-OH-XYL), of lidocaine in human urine samples after acid hydrolysis of its conjugates. Detemination of 4-OH-XYL was performed using micro extraction in packed syringe (MEPS) as a sample preparation method on-line with liquid chromatography and tandem mass spectrometry (MEPS-LC-MS-MS). MEPS is a new technique for miniaturised solid-phase extraction that can be connected, online, to gas chromatography (GC) or liquid chromatography (LC) without any modifications. The validation of the method was performed in the range 17 to 8700 nmol/L. The inter-day accuracy and precision values were determined from six replicates of quality control (QC) samples at three different concentrations in human urine. The nominal urine concentrations of 4-OH-XYL in the QC samples were 80.5, 805, and 8050 nmol/L. The mean accuracy for each concentration level, reported as the percentage difference from the nominal value, was within ±8%. The precisions, given as coefficient of variation, were in the range 6 to 8%. The validated method was used for the quantification of 4-OH-XYL in urine samples from subjects receiving lidocaine.

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It was found that 4-OH-XYL is stable under highly acidic conditions (pH $\leq$ 1), and is highly unstable in neutral and alkaline solutions. Our investigation showed that 4-OH-XYL is stable for at least 24 hours in the refrigerator in 0.1 M HCl (pH $\approx$ 1). At pH 9.1, more than 40% decomposition of 4-OH-XYL occurs within 6 h at room temperature compared to 20% at pH 2.0. Therefore, our approach was to use acid hydrolysis of 4-OH-XYL conjugates and on-line sample preparation for the quantification of 4-OH-XYL in humane urine.

Keywords: Column liquid chromatography-tandem mass spectrometry, Urine samples, Microextraction in packed syringe, Sample preparation, 4-OH-XYL

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INTRODUCTION

Lidocaine is a commonly used amide type local anesthetic agent that has been on the market for over half a century. Lidocaine also has antiarrhythmic properties and is used as a therapeutic agent in the treatment of cardiac disorders. The pharmacokinetics of lidocaine have been studied extensively, both in animals and man.[1] The metabolism of lidocaine in man has been a subject of interest for a number of years. The major metabolites of lidocaine are monoethylglycinexylidide (MEGX) and glycinexylidide (GX), 2,6-xylidine (XYL) and 4-OH-2,6-xylidine (4-OH-XYL), with the later being reported to be the most abundant metabolite in both plasma and urine.[1,2,7] After administration, lidocaine is mainly excreted in the urine with 4-OH-XYL found in up to 70–80% of the dose in the form of its glucuronide and/or sulfate conjugates.[1,2,7]

The determination of lidocaine and its metabolites have been performed by liquid and gas chromatography, spectrophotometry, and fluorometry.[1–12] In a previous study we published a simple and sensitive quantification method for lidocaine and three of its metabolites (GX, MEGX and XYL) using reversed phase LC and tandem mass spectrometric detection.[3] The quantification of 4-OH-XYL is a challenge due to the instability of this compound, and various acid and enzyme hydrolysis followed by derivatization have been developed to study the conjugates 4-OH-XYL.[5,7]

In this study we have developed a selective method for the quantification of 4-OH-XYL, without prior derivatization, in human urine after acid hydrolysis of its conjugate, using microextraction in packed syringe (MEPS) as sample preparation on-line with LC-MS-MS.

MEPS (Fig. 1A) is a new miniaturized solid-phase extraction method that can be connected on-line to GC or LC without any modifications.[13–16] In the MEPS technique, approximately 1 mg of the solid packing material is inserted inside a syringe (100–250 μL) as a plug inside the syringe or between the barrel and the needle. Sample extraction takes place on the packed bed. The bed can be coated to provide selective and suitable sampling conditions. This approach to sample analysis is very promising for many reasons: 1) It is easy to use, 2) Fully automated on-line procedure,

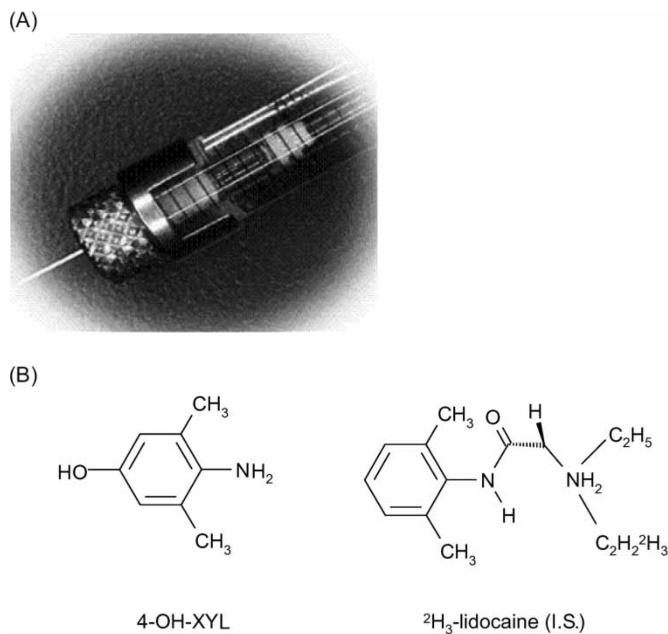

Figure 1. (A) Microextraction in packed syringe (MEPS). (B) Chemical structure 4-OH-2,6-xylidine and $^2H_3$-lidocaine (I.S.).

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3) Rapid, and 4) The cost of analysis is minimal when compared to conventional solid phase extraction.

EXPERIMENTAL

Chemicals

4-OH-XYL and $^2H_3$-lidocaine, IS (Fig. 1B) were supplied by the Department of Chemistry, AstraZeneca R&D (Södertälje, Sweden). Acetonitrile, methanol, formic acid, and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Apparatus

An LC-instrument with two Shimadzu LC10Advp pumps (Kyoto, Japan) was connected to an autosampler, CTC-Pal from Crelab (Knivsta, Sweden) and a 20 μL sample loop. A Symmetry C18, 5 μm (150 × 2.1 mm) column obtained from Waters (MA, USA) was used as the analytical column. A Valco C4W valve from Valco Instruments (Houston, USA) was used as a

gate valve between the LC system and the mass spectrometer. The Milli-Q water was obtained using a reagent grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, USA). A gradient LC pump was used with mixer volume of 0.1 mL. Mobile phase A was 0.1% formic acid in acetonitrile + water, 10 + 90 (v/v) and mobile phase B contained 0.1% formic acid in acetonitrile + water, 80 + 20 (v/v). The gradient started from 0% of phase B up to 80% from 1 to 5 min and then from 5 to 6 min isocratic at 80% of phase B, and at 6.1 min phase B was set at 0% again. For system stability the next injection was performed after 8 min. The flow rate was 200 μL/min.

The mass spectrometry (MS) was performed with a Quattro triple quadrupole mass spectrometer (Quattro II, Micromass, Manchester, UK) equipped with a Z-electrospray interface (ESI) operated in positive ion mode. The electrospray interface was maintained at 150°C. Nitrogen was used as drying and nebulizer gases. Argon was used as collision gas. The parameter settings used were: capillary voltage at 3.1 kV, cone voltage at 38 V, extractor at 5 V, RF lens at 0.2 V, source block and desolvation temperatures at 150°C and 300°C, respectively. Nitrogen was used both as drying (400 L h^{-1}), and nebulizing gases (20 L h^{-1}), the vacuum was $2 \cdot 10^{-5}$ in the mass analyzer and $2 \cdot 10^{-3}$ in the collision cell. Argon was used as collision gas and collision energy was 25 eV. The gases were from SanGas (Stockholm, Sweden). The data were collected and processed using MassLynx version 3.5, and all calculations were based on peak area ratios. The scan mode was multiple reaction monitoring (MRM) using the precursor ion at $[M + H]^+$ (*m/z:* 138 and 238), and after collisional dissociation the product ions at m/z: 77 and 88 were used for quantification of 4-OH-XYL and internal standard, respectively.

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Preparation of Standard Solutions and Acid Hydrolysis

Stock Solutions

Two stock solutions of 4-OH-XYL in methanol were prepared (one for standards and one for quality control samples). From the stock solution stepwise dilution series were made. Spiked urine samples were prepared by adding 5–50 μL of analyte standard to 1.0 mL urine. The concentration range of the calibration curve was between 17–8700 nmol/L.

Acid Hydrolysis

One hundred μL of patient, obtained from an AstraZeneca in-house mass balance study of lidocaine in man, or spiked urine sample was added to an equal volume of 6 M HCl. All samples were incubated at 90°C for 2 h. After hydrolysis 20 μL acid hydrolysed urine was added to 980 μL water and 20 μL internal standard in vials. Patients' urine samples were stored at

−20°C, prior to analysis. Before use, the urine was thawed at room temperature and centrifuged at +4°C.

MEPS Conditions

MEPS was performed using a 250 μL gas tight syringe. The sorbent used was a polystyrene polymer, ISOLUTE ENV+, from Argonaut (Mid Glamorgan, United Kingdom). This sorbent has irregular particles with an average size of 50 μm and nominal 60 Å porosity. One milligram of the solid material was manually inserted inside the syringe as a plug. The sorbent material was tightened by filters in order to avoid moving inside the syringe.

Before use, the sorbent was manually conditioned with 50 μL methanol followed by 50 μL of water/methanol, 90:10 (v/v). After that, the syringe was connected to the auto sampler and the urine sample (50 μL) was introduced into the syringe by the auto sampler. The sorbent was then washed once with 100 μL of washing solution. The analytes were then desorbed by 20 μL with elution solution directly into a gate valve situated between the LC and MS. Cleaning of the sorbent was carried out using 5 × 50 μL elution solution, followed by 5 × 50 μL of the washing solution between every extraction. This step decreased memory effects, but also functioned as a conditioning step before the next extraction. The same packing bed was used for about 100 extractions before it was discarded. Water/methanol (90 : 10, v/v) and 0.25% ammonium hydroxide in methanol/water (95 : 5, v/v) were used as washing and elution solutions, respectively.

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Method Validation

Calibration samples solutions (8 concentrations) in human urine samples were prepared within a concentration range of approximately 17–8700 nmol/L. A calibration curve with eight standard concentrations was prepared. The quality control (QC) samples in human urine were prepared with the concentrations of 80.5, 805, and 8050 nmol/L. The accuracy and precision were calculated for the QC samples at three different occasions. The method was validated at optimized conditions.

The ratios of peak areas of 4-OH-XYL and the internal standard were measured and a standard curve without the zero concentration was constructed. Calibration curves were typically described by the equation:

$$y = Ax^2 + Bx + C,$$

where y is the peak height ratio, x is the concentration of analyte, and B and C are the slope and intercept, respectively, and A is the curvature. The calibration curves were weighted (1/x). Accuracy was defined as the degree of

deviation of the determined value and the nominal value: [(determined value − nominal value)/nominal value] × 100. Precision (C.V.%) was defined as the percentage of standard deviation of the observed values divided by their mean values: [(Standard deviation)/mean value] × 100.

The lower limit of quantification (LLOQ) was set as the lowest measurable concentration with acceptable accuracy and precision (should not exceed 20% CV and $S/N \geq 10$, peak to peak). Method selectivity was defined as non-interference with the endogenous substances in the regions of interest.

Stability

The stability of 4-OH-XYL at different pH solutions (1, 2, 5.8, and 9.1) was investigated.

Also, different hydrolysis times (30, 60, 120, and 180 min) were investigated at 90°C.

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RESULTS AND DISCUSSION

Stability of 4-OH-XYL

The quantification of unconjugated 4-OH-XYL in plasma or urine samples has been shown to be difficult due to instability of this compound in neutral and alkaline solution. The mechanism of 4-OH-XYL decomposition has been studied by others,[17] and it was found that 4-OH-XYL is oxidized to the corresponding *p*-quinine via the intermediate imine. This reaction requires oxygen and is highly pH dependent. The stability of 4-OH-XYL rapidly decreases as pH increases above pH 2 (Fig. 2). Our investigation showed that the concentration of 4-OH-XYL was decreased by about 40% after 6 h at room temperature at pH 9.1, compared to 20% at pH 2.0. It was found that the 4-OH-XYL completely disappeared after 72 h at pH 9.1 at room temperature (Fig. 2). At pH 5.8 the instability of 4-OH-XYL is high, the concentration of 4-OH-XYL decreased by ca 30% after 4 h. However, we found that the 4-OH-XYL is stable for at least 24 h in 0.1 M HCl ($pH \approx 1$). Antioxidants have been used to stabilize 4-OH-XYL, however, the results are very variable.[18] Another approach to stabilize 4-OH-XYL is to remove the oxygen by purging nitrogen in the aqueous sample, however, we found that under high pH (≈ 7) the stability was not improved (may be due to the pH effect on the stability of 4-OH-XYL).

The investigation of the stability suggested to us that in order to quantify 4-OH-XYL and its conjugates, the acid hydrolysis method is the method of choice followed by a direct injection of the acidic sample, without neutralization, into the LC-MS/MS. We also adopted our new on-line sample preparation technique, MEPS (microextraction in packed syringe), to wash away HCl and

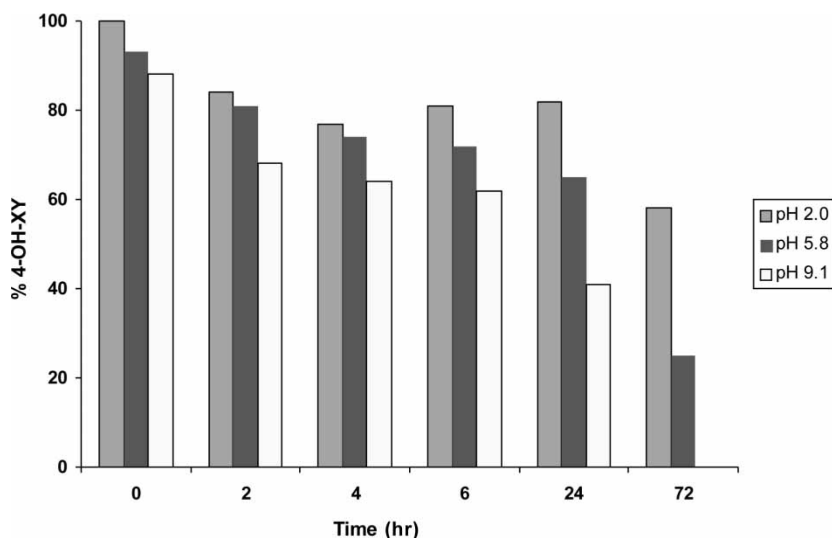

Figure 2. The stability of 4-OH-2,6-xylidine in different pH solutions.

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directly inject the hydrolysed sample. Different hydrolysis times (30–180 min) were investigated at 90°C and the optimum time was 2 h.

MEPS Conditions: Washing and Elution Solvents

To optimize micro extraction in packed syringe, factors affecting the recovery such as the composition of washing solution, and elution solutions were studied.

After introducing the sample (50 μL) into the syringe, it was washed once with 100 μL of 5% methanol in water to remove salts and HCl. Solutions containing methanol, water, formic acid, and ammonium hydroxide were investigated as elution solvents. After washing with 100 μL of water, the elution efficiency was measured and compared to that of a pure standard solution (1000 nmol/L). Eluting efficiency increased significantly with increasing methanol content in the eluent, while the use of formic acid or ammonium hydroxide did not affect the recovery of the drug. Acceptable recovery ($50 \pm 3\%$) and clean extract were obtained when a solution of methanol/water, 95 : 5 (v/v) with 0.25% ammonium hydroxide was used as elution solution.

Selectivity

No significant interference was detected in chromatograms obtained from blank urine (see Fig. 3A and B). The lowest calibration concentration was set to about 17 nmol/L.

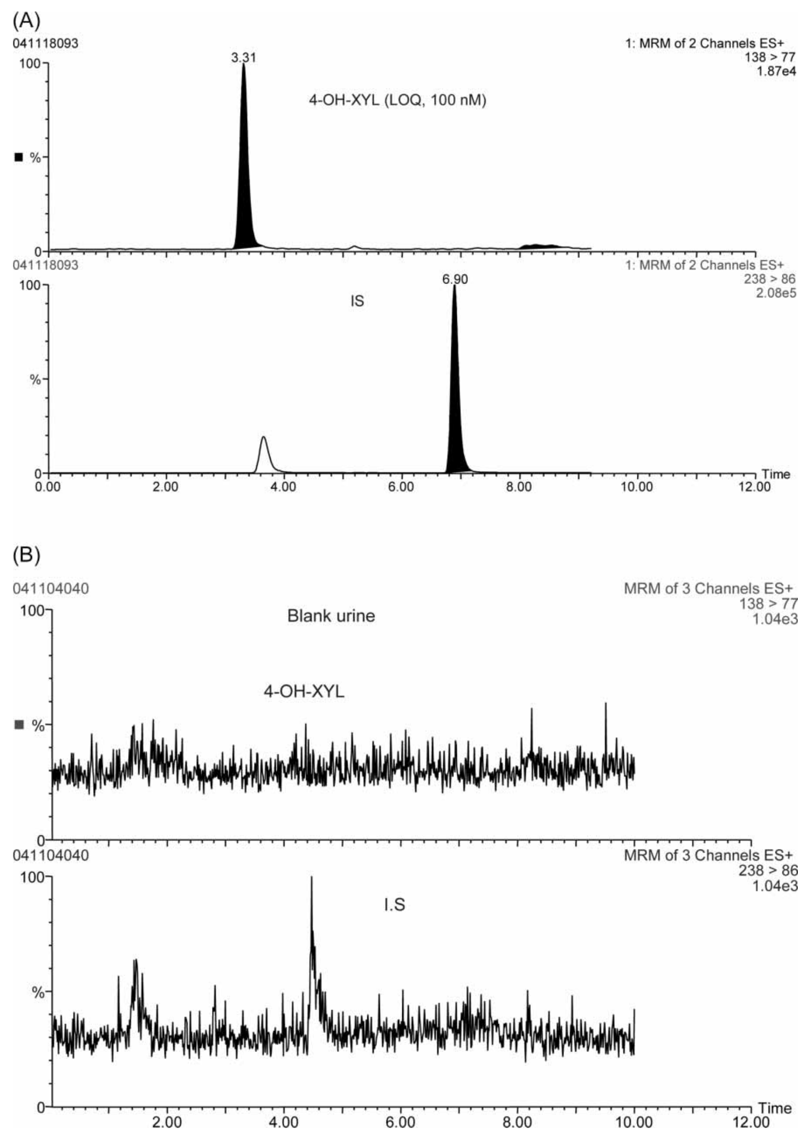

Figure 3. Representative chromatograms with mass spectrometric detection obtained from (A) human urine spiked with 4-OH-2,6-xylidine 100 nmol/L (LLOQ) and $^{2}H_{3}$-lidocaine as internal standard (I.S.); (B) blank urine sample.

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Calibration Data

$^{2}H_{3}$-lidocaine was used as internal standard to validate the method. The constructed calibration curve for urine samples consisted of eight levels of spiked human urine in the concentration range 17–8700 nmol/L. The regression

Table 1. Regression parameters for calibration curves of 4-OH-XYL in diluted urine at three different assays

Assay	Curvature *a* (10^{-7})	Slope *b*	Intercept *c*	R^2
(A)	0.13	0.0005	0.0017	0.9999
(B)	−0.35	0.0009	0.0011	0.9997
(C)	−0.57	0.0008	0.0032	0.9997

correlation coefficients (R^2) were over 0.999 in all experiments (Table 1). The back calculated values of the calibration points showed good agreement with the theoretical concentrations, deviation between 0% and −14% of the nominal concentrations was observed (n = 3).

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Accuracy and Precision

The nominal urine concentrations of 4-OH-XYL in the QC samples were 80.5, 805, and 8050 nmol/L. The intra-day precisions, given as coefficient of variation (CV), at three different concentrations for QC samples were about 4–8% (n = 6). For the inter-day the mean accuracies, reported as the percentage difference from the nominal concentration (n = 18), were −5.5, −6.8, and 7.4%, respectively. The inter-day precisions (CV), were 8, 7, and 6% at 80.5, 805, and 8050 nmol/L, respectively. The accuracy and precision data are summarized in Table 2.

Application of the Method

The method was applied for the analysis of real urine samples from different subjects. Figure 4 shows a mass chromatogram of patient's urine sample (planned dose: 4 mg/kg, planned time: 4–6 h).

Table 2. Intra- and inter-day precision and accuracy for 4-OH-XYL in urine (QC samples)

Concentration (nmol/L)	Inter-day accuracy[a] (%) (n = 18)	Intra-day precision (%CV) (n = 6)	Inter-day precision (%CV) (n = 18)
80.5	−5.5	8.0	8.0
805	−6.8	6.0	7.0
8050	7.4	4.0	6.0

[a]The percentage difference from the nominal value.

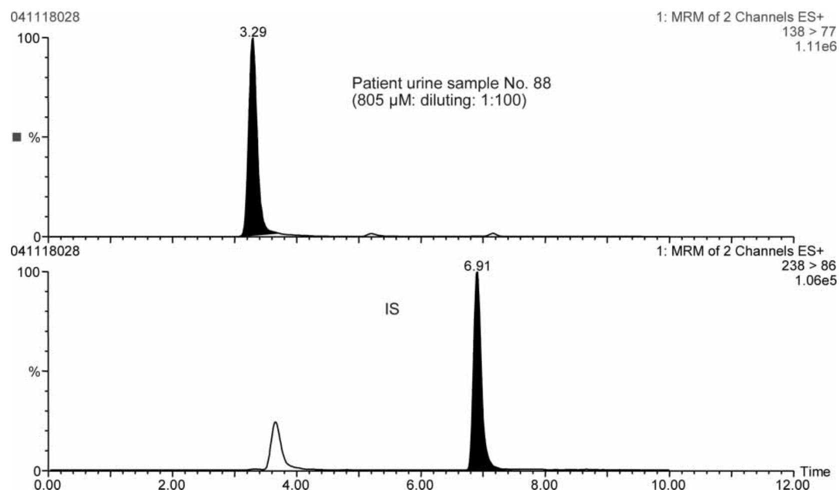

Figure 4. Mass chromatogram obtained from urine sample collected from subject 1 (planned dose: 4 mg/kg, planned time: 4–6 h); sample No 88 (805 μmol/L) with 4-OH-2,6-xylidine and $^2H_3$-lidocaine as internal standard (I.S.).

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Lower Limit of Quantification (LLOQ)

The lower limit of quantification was set to 100 nmol/L for the analysis of human urine samples from clinical studies (dose: 4 mg/kg). The mean accuracy of LOQ, reported as the percentage difference from the nominal concentration, was −5% (n = 6). The precision, given as coefficient of variation (CV), was 8% (n = 6).

CONCLUSIONS

An LC-MS/MS method for the assay of 4-OH-XYL, the major human metabolite of lidocaine, in urine samples has been developed and validated. The acceptance criteria for the study validation were well in line with the international criteria.[19] The results showed that the method is selective and accurate. Micro extraction in packed syringe is a new sample preparation method suitable for the fully automated determination of analytes in complex matrices. It was thus shown that it was suitable for unstable compounds and is rapid and selective.

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